Anesthesiology 2006; 105:726–36

New Insights into the Mechanism of Methoxyflurane Nephrotoxicity and Implications for Anesthetic Development (Part 1)

Identification of the Nephrotoxic Metabolic Pathway

Evan D. Kharasch, M.D., Ph.D.,* Jesara L. Schroeder, B.S.,† H. Denny Liggitt, D.V.M., Ph.D.,‡ Sang B. Park, Ph.D.,§ Dale Whittington, B.S.,§ Pamela Sheffels, B.S.§

Background: **Methoxyflurane nephrotoxicity results from biotransformation; inorganic fluoride is a toxic metabolite. Concern exists about potential renal toxicity from volatile anesthetic defluorination, but many anesthetics increase fluoride concentrations without consequence. Methoxyflurane is metabolized by both dechlorination to methoxydifluoroacetic acid (MDFA, which may degrade to fluoride) and O-demethylation to fluoride and dichloroacetatic acid. The metabolic pathway responsible for methoxyflurane nephrotoxicity has not, however, been identified, which was the aim of this investigation.**

Methods: **Experiments evaluated methoxyflurane metabolite formation and effects of enzyme induction or inhibition on methoxyflurane metabolism and toxicity. Rats pretreated with phenobarbital, barium sulfate, or nothing were anesthetized with methoxyflurane, and renal function and urine methoxyflurane metabolite excretion were assessed. Phenobarbital effects on MDFA metabolism and toxicity *in vivo* were also assessed. Metabolism of methoxyflurane and MDFA in microsomes from livers of pretreated rats was determined *in vitro*.**

Results: **Phenobarbital pretreatment increased methoxyflurane nephrotoxicity *in vivo* (increased diuresis and blood urea nitrogen and decreased urine osmolality) and induced *in vitro* hepatic microsomal methoxyflurane metabolism to inorganic fluoride (2-fold), dichloroacetatic acid (1.5-fold), and MDFA (5-fold). In contrast, phenobarbital had no influence on MDFA renal effects *in vivo* or MDFA metabolism *in vitro* or *in vivo*. MDFA was neither metabolized to fluoride nor nephrotoxic. Barium sulfate diminished methoxyflurane metabolism and nephrotoxicity *in vivo*.**

Conclusions: **Fluoride from methoxyflurane anesthesia derives from O-demethylation. Phenobarbital increases in methoxyflurane toxicity do not seem attributable to methoxyflurane dechlorination, MDFA toxicity, or MDFA metabolism to another toxic metabolite, suggesting that nephrotoxicity is attributable to methoxyflurane O-demethylation. Fluoride, one of many metabolites from O-demethylation, may be toxic and/or reflect formation of a different toxic metabolite. These results may have implications for interpreting anesthetic defluorination, volatile anesthetic use, and methods to evaluate anesthetic toxicity.**

* Russell D. and Mary B. Sheldon Professor of Anesthesiology and Director, Division of Clinical and Translational Research, Department of Anesthesiology, Washington University. † Research Technologist, § Research Scientist, Department of Anesthesiology, ‡ Professor and Chair, Department of Comparative Medicine, University of Washington.

Received from the Departments of Anesthesiology, Washington University, St. Louis, Missouri, and University of Washington, Seattle, Washington. Submitted for publication October 26, 2005. Accepted for publication June 2, 2006. Supported by grant Nos. R01DK53765 and P30ES07033 from the National Institutes of Health, Bethesda, Maryland.

Address correspondence to Dr. Kharasch: Division of Clinical and Translational Research, Department of Anesthesiology, Washington University, 660 South Euclid Avenue, Campus Box 8054, St. Louis, Missouri 63110-1093. kharasch@wustl.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

FOR nearly half a century, the potential for nephrotoxicity has influenced the pharmaceutical development and clinical use of volatile anesthetics. This concern is grounded in the experience with methoxyflurane, introduced in 1960,[1] which caused dose-related subclinical or overt renal insufficiency in humans and animals.[2,3] Toxicity was causally related to methoxyflurane metabolism and associated with increased plasma fluoride concentrations. In the intervening decades, every prospective new volatile anesthetic has been experimentally and clinically evaluated for its potential to undergo metabolism, increase plasma fluoride concentrations, and purportedly cause nephrotoxicity. Some candidates might have been rejected simply because they underwent defluorination. Others, such as sevoflurane, were delayed for years.[4,5]

There is unambiguous evidence, based in part on the seminal contributions of Richard Mazze[3] and others, that methoxyflurane nephrotoxicity is related to its metabolism. Methoxyflurane undergoes oxidative metabolism by cytochrome P450, with liberation of inorganic fluoride ion as a major metabolite.[6] Soon after recognition of methoxyflurane nephrotoxicity, an association with increased plasma fluoride concentrations was noted, and it was suggested that fluoride was the causative nephrotoxin.[7,8] In animals, increased plasma fluoride concentrations were associated with structural and functional renal abnormalities.[9] Both plasma fluoride concentrations and nephrotoxicity increased as a function of methoxyflurane dose.[10] In animals, induction of P450 enzymes by phenobarbital pretreatment increased methoxyflurane metabolism, accentuated the increase in plasma fluoride concentration, and worsened nephrotoxicity, whereas P450 inhibition decreased metabolism, plasma fluoride, and renal toxicity.[11–13] Injection of animals with inorganic fluoride, albeit at very large doses, produced changes in renal function and morphology similar to those seen after methoxyflurane.[9,10,12] Clinically, there were methoxyflurane dose-related abnormalities in renal function in surgical patients.[3] In addition, there was an association with serum fluoride concentrations. No renal abnormalities were reported at peak fluoride concentrations of less than 40 μM, subclinical toxicity was accompanied by peak fluoride of 50–80 μM, mild clinical toxicity was associated with peak fluoride of 90–120 μM, and overt nephrotoxicity was associated with peak fluoride concentrations of 80–175 μM.[14]

Based on these associations, it was concluded that methoxyflurane nephrotoxicity was caused by inorganic fluoride. The once classically accepted "fluoride hypothesis," subsequently generalized to volatile anesthetics in general (albeit without any evidence), was that anesthetics undergo hepatic defluorination, that inorganic fluoride is released and disseminated systemically and acts as a specific renal toxin, and that the threshold for renal toxicity is plasma fluoride concentrations exceeding 50 μM. Nevertheless, it is now clear that hepatic defluorination and systemic translocation of fluoride to the kidney is not the exclusive mechanism of anesthetic toxicity, that a generic plasma fluoride concentration of greater than 50 μM has no causal implications for renal toxicity, and that the 50-μM fluoride threshold for nephrotoxicity has been relegated to historical artifact.[15–17] Numerous investigations have demonstrated the absence of renal toxicity with enflurane, isoflurane, and sevoflurane despite plasma fluoride concentrations exceeding 50 μM or even 100 μM, and neither the peak systemic fluoride concentration nor the duration of fluoride increase alone can be applied nonselectively to all anesthetics to explain or predict nephrotoxicity.[15] Rather, anesthetic nephrotoxicity seems agent (*i.e.*, methoxyflurane) specific, the role of fluoride in methoxyflurane toxicity is incompletely understood, and the mechanisms of methoxyflurane toxicity remain enigmatic.

Why, then, is methoxyflurane nephrotoxic, how is toxicity related to metabolism, and why was the toxicity associated with a plasma fluoride concentration of greater than 50 μM? And, given that methoxyflurane disappeared from clinical use decades ago, why are these questions important? One potential explanation for methoxyflurane nephrotoxicity is that intrarenal production of fluoride (or other toxins) may be the actual determinant of anesthetic toxicity. A previous investigation showed that human kidney microsomes substantially defluorinated methoxyflurane but not nonnephrotoxic anesthetics.[15] Another (not necessarily exclusive) explanation is that a different methoxyflurane metabolite (unique to methoxyflurane) may be the actual toxin. If so, coformed inorganic fluoride would simply be a marker for formation of the actual nephrotoxin—an epiphenomenon. All of the above animal and human experimental and clinical observations could also be explained by a nephrotoxic methoxyflurane metabolite coformed with fluoride.

Methoxyflurane undergoes defluorination by two different routes (fig. 1).[6,18] Oxidative dechlorination of the chloromethyl carbon yields 2,2-difluoro-2-methoxyacetic acid (which may degrade to fluoride). Oxidative O-demethylation of the methoxy group yields fluoride and dichloroacetatic acid (thought in turn to yield oxalic acid). Both metabolic pathways may form inorganic fluoride. Nevertheless, the metabolic pathway that is responsible for methoxyflurane nephrotoxicity has never been identified.

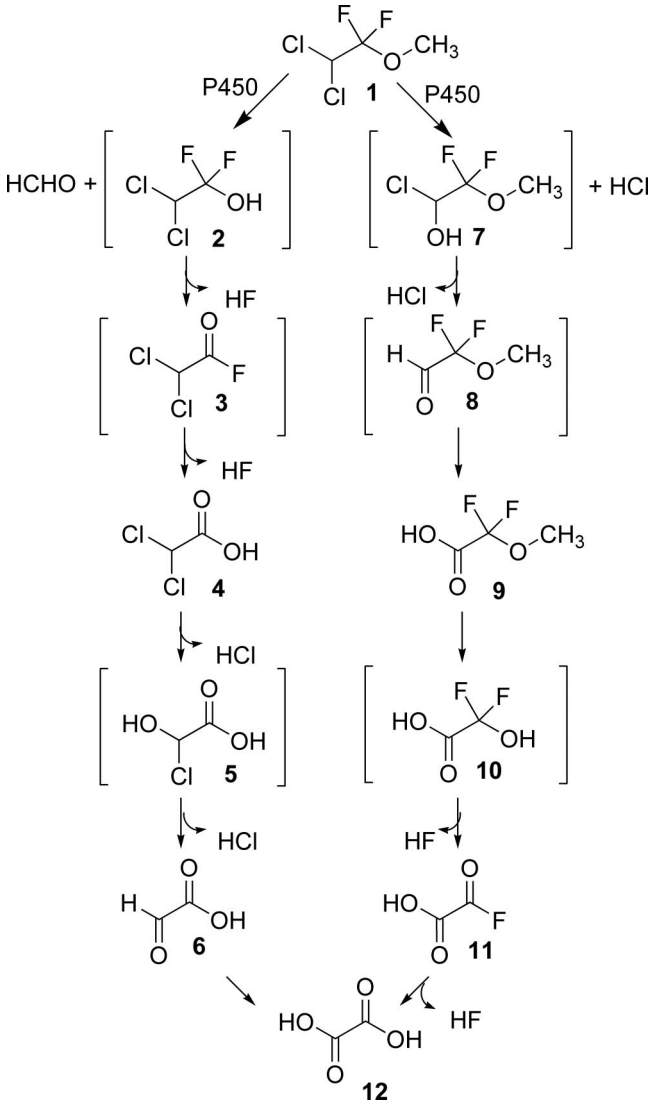

Fig. 1. Proposed pathways of methoxyflurane metabolism.[8] Proposed or unstable intermediates are shown in *brackets*. The O-demethylation pathway is on the *left*, and the dechlorination pathway is on the *right*. *1* = methoxyflurane; *2* = 2,2 dichloro-1,1difluoroethanol; *3* = 2,2 dichloroacetylfluoride; *4* = dichloroacetic acid (DCAA); *5* = hydroxychloroacetic acid; *6* = glyoxalic acid; 7 = 2-chloro-1,1-difluoro-2-hydroxy-1-methoxyethane; *8* = 2,2-difluoro-2-methoxyacetaldehyde; *9* = 2,2-difluoro-2-methoxyacetic acid (methoxydifluoroacetic acid [MDFA]); *10* = difluorohydroxyacetic acid; *11* = monoxalyl fluoride; *12* = oxalic acid.

The purpose of this investigation was to identify which pathway of methoxyflurane metabolism mediates nephrotoxicity in rats. Experiments were conducted to quantify formation of methoxyflurane metabolites and evaluate the effect of enzyme induction or inhibition on both methoxyflurane metabolism and toxicity.

Materials and Methods

Chemicals

Methoxyflurane was obtained from Abbott Laboratories (Abbott Park, IL). Identity and purity were confirmed by

gas chromatography–mass spectrometry (GC-MS). Dichloroacetic acid (DCAA), barium sulfate, and phenobarbital were purchased from Sigma Chemical Company (St. Louis, MO). Trifluoroacetic acid, Magtrieve, and benzophenone hydrazone were purchased from Aldrich (Milwaukee, WI). Chlorodifluoroacetic acid was purchased from Fluka (Milwaukee, WI). Methylene chloride and hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). Ethyl acetate (GC grade) was obtained from Burdick and Jackson (Muskegon, MI). Anhydrous ether was from J.T. Baker (Phillipsburg, NJ). 2,2-Difluoro-2-methoxyacetic acid (methoxydifluoroacetic acid [MDFA]) was synthesized as described previously[19] and was 99% pure.

Animals and Treatments

All experiments were approved by the University of Washington Animal Care and Use Committee, Seattle, Washington, and conducted in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Male Fischer 344 rats (12 weeks old, 250–300 g) were purchased from Harlan (San Diego, CA). Rats were housed in individual metabolic cages, provided food and water *ad libitum*, and maintained on a 12-h light–dark cycle. They were allowed to acclimate to the metabolism cages for at least 48 h before any experimental treatment. Before any treatment, a baseline 24-h urine collection and blood sample *via* saphenous vein puncture were obtained (300 μl collected in capillary tubes containing EDTA; Sarstedt, Newton, NC). Blood was centrifuged and plasma stored at −80°C. All urine samples were also stored at −80°C.

To evaluate methoxyflurane metabolism and toxicity, animals were anesthetized for 3 h with 0.5% methoxyflurane (1.3 times the minimum alveolar concentration) in oxygen[9,10,20] at 8 l/min *via* a calibrated methoxyflurane vaporizer, in a 20-l inhalation chamber without the use of carbon dioxide absorbent. Animals rested on a warming pad, with constant monitoring to maintain rectal temperature at 37°C. Chamber gas concentrations were monitored constantly to maintain the desired methoxyflurane concentration and minimize any carbon dioxide rebreathing (Capnomac Ultima; Datex, Madison, WI). Two methods were used to monitor methoxyflurane concentrations. Real-time monitoring was *via* the sevoflurane channel on the Capnomac, which was calibrated using methoxyflurane standards whose concentration was determined by GC-MS, as described in Analytical Methods, below. For example, 0.5% methoxyflurane yielded a Capnomac monitor reading of "0.65% sevoflurane." In addition, gas samples (100 μl) were removed from the chamber every 30 min using a gastight syringe and injected into a sealed headspace vial, and concentrations were determined by GC-MS. After recovery from anesthesia, animals were returned to the metabolic cages, and blood and 24-h urine samples were obtained daily for 4 days. Blood was obtained from saphenous and tail veins on alternate days. Blood was centrifuged and plasma stored at −20°C until analyzed. Urine was stored at −80°C until analyzed. On the fifth day, rats were killed, the kidneys were excised, a transverse section of the kidney was preserved in 10% formalin for histologic analysis, and the remainder was flash frozen in liquid nitrogen and stored at −80°C.

Experiments were conducted to determine the effect of enzyme induction or inhibition on methoxyflurane metabolism and renal toxicity. To determine the effect of phenobarbital, rats (n = 5) received phenobarbital (80 mg/kg *via* intraperitoneal injection in 25 mg/ml saline) for 4 days. Controls (n = 4) received saline only (2 ml/kg) for 4 days. One day after the last dose of phenobarbital, all rats were anesthetized with methoxyflurane as described in Animals and Treatments. To test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity, an experiment was attempted to manipulate selectively renal P450 activity and metabolism, without altering hepatic metabolism or systemic fluoride concentrations. Barium sulfate was reported to significantly increase renal cytochrome P4502E1 activity (a major P450 isoform responsible for methoxyflurane metabolism[21]) and decrease total hepatic P450 content.[22] Therefore, the effect of barium sulfate on methoxyflurane metabolism and nephrotoxicity was evaluated. Rats received barium sulfate (4 g/kg *via* intraperitoneal injection in 0.5 g/ml saline; n = 10) or saline (8 ml/kg; n = 8) 48 h before methoxyflurane exposure.[22]

To determine the effect of enzyme activity on MDFA metabolism and toxicity, rats were randomly assigned to pretreatment with phenobarbital (0.1% in drinking water for 10 days before MDFA dosing and thereafter) or nothing (n = 4). The dose regimen for MDFA was based on preliminary experiments with inhaled methoxyflurane, showing the greatest metabolite excretion on day 1 or 2, an average of half that excretion on subsequent days, and 20–100 μmol MDFA excreted per day (*vide infra*, and other results not shown). Because the effect of phenobarbital on MDFA metabolism and toxicity was unknown, as was the extent to which MDFA excretion reflected MDFA formation, half of the excreted dose was injected. All animals received MDFA (0.225 mmol/kg *via* intraperitoneal injection in saline, followed by 0.112 mmol/kg daily for 3 days). Daily blood and urine samples were obtained as described above.

In Vitro *Metabolism*

Livers were obtained from rats treated with phenobarbital (0.1% in drinking water for 10 days), barium sulfate (4 g/kg *via* intraperitoneal injection 48 h before death), or saline, perfused with ice-cold phosphate-buffered saline (pH 7.4), and immediately frozen and stored at

−80°C until used to prepare microsomes, as described previously.[15] Incubations (1 ml) contained 3 mg liver or kidney microsomal protein in 50 mM Tris-HCl buffer (pH 7.4), and 2 μl methoxyflurane and nicotinamide adenine dinucleotide phosphate, reduced (final concentration 2 mM), were added to start the reaction, as described previously.[15] Reactions were terminated after 30 min by flash freezing with liquid nitrogen. Incubations with MDFA (0.5 mM) were performed in a similar manner. Concentrations of fluoride, MDFA, and DCAA in the quenched reactions were determined as described below, in Analytical Methods.

Analytical Methods

Concentrations of fluoride in urine, blood urea nitrogen (BUN), and urine osmolality were determined as described previously.[23] Concentrations of fluoride in microsomal incubations were determined by a fluoride-specific electrode, using standard curves of known fluoride standards and microsomes, as described previously.[15] Plasma creatinine concentrations were measured using Vitros Crea slides on a Vitros 250 chemistry system (Ortho-Clinical Diagnostics, Rochester, NY). Kidneys were fixed in 10% neutral-buffered formalin and processed, and histopathologic analysis was performed by a veterinary pathologist who was blinded to animal treatments as described previously.[23] The semiquantitative severity score ranged from 0 to 4 (normal, minimal, slight, moderate, and marked, respectively) to reflect the degree and distribution of any tubular necrosis.

Methoxyflurane concentrations in the inhalation chamber were determined by GC-MS using an Agilent (Palo Alto, CA) 6890-5973 with 7694 headspace autosampler containing a DB-VRX column (30 m × 0.32 mm × 1.8 μm film thickness; J&W Scientific, Folsom, CA). The headspace sample oven, sample valve, and headspace transfer line were 50°, 60° and 70°C, respectively. The parameters for the headspace for sampling were as follows: agitation = high; sample equilibration time = 1.0 min; vial pressurization time = 0.1 min; vial pressure = 18 psi; loop fill time = 0.25 min; loop equilibration time = 0.15 min; sample inject time = 0.25 min; and oven stabilization time = 1.0 min. The gas chromatograph inlet was operated in split mode (split ratio 5:1) with the split flow at 10 ml/min and the total flow at 15 ml/min. The inlet and transfer line temperatures were 150° and 250°C, respectively. The helium carrier gas was delivered in constant-flow mode at 2 ml/min. The initial oven temperature was 100°C, which was held for 2 min, then increased to 185°C at 40°C/min, and held for 3 min. Methoxyflurane eluted at 5.4 min and was detected by selected-ion monitoring (*m/z* 81). Methoxyflurane was quantified using a standard curve (range 0.43–1.7% methoxyflurane).

MDFA and DCAA concentrations in urine and microsomal incubations were determined by GC-MS. Rat urine (unknown, calibration, or quality control samples, 20 μl) was added to 16 × 125-mm glass tubes, followed by the internal standards trifluoroacetic acid and chlorodifluoroacetic acid (100 μl of 4 mg/ml; 400 μg of each standard), and 250 μl of 0.1N HCl, and vortexed. Samples were twice extracted with 1 ml diethyl ether and vortexing, followed by centrifugation at 700*g* for 10 min. The organic layers were removed and combined, and the diphenyldiazomethane derivitizing agent (75 μl, prepared as described previously[23]) was added and vortexed. After 45 min, 20 μl methanol was added to quench the reaction. Samples were transferred into autosampler vials and sealed for GC-MS analysis, using the same instrument as for methoxyflurane analysis above, but equipped with a DB-5 fused-silica capillary column (30 m × 0.32 mm × 0.5 μm; J&W Scientific). The helium carrier gas was delivered at a constant flow of 1.3 ml/min. Samples (2 μl) were injected using an Agilent 7983 autosampler. The injector and transfer line temperatures were 250° and 300°C, respectively. The oven was held at 50°C for 1 min, increased at 25°C/min to 250°C, held for 1 min, increased at 35°C/min to 300°C, and held for 6 min. Analytes were detected using selected-ion monitoring for MDFA (*m/z* 292, 11.2 min), DCAA (*m/z* 294, 12.5 min), trifluoroacetic acid (*m/z* 280, 8.7 min), and chlorodifluoroacetic acid (*m/z* 296, 10.3 min) and quantified using standard curves of peak area ratios (MDFA/trifluoroacetic acid and DCAA/chlorodifluoroacetic acid) *versus* analyte concentration. Calibration curves were obtained by analyzing blank rat urine containing MDFA and DCAA at 100, 250, 500, 750, 1,000, 2,500, and 5,000 μg/ml. Quality control samples (250, 750, 2,500 μg/ml) of MDFA and DCAA were also analyzed. Coefficients of variation were 6–9% for both analytes.

Statistical Analysis

Data were analyzed using two-way repeated-measures analysis of variance (time and treatment), followed by the Student-Newman-Keuls test for individual comparisons (within group days 1–4 *vs.* baseline, and between treatments), using SigmaStat (Systat, Point Richmond, CA). All results are reported as mean ± SD. Statistical significance was assigned at $P < 0.05$.

Results

Experiments were conducted to determine the mechanism by which pretreatment with phenobarbital, an inducer of multiple hepatic and renal cytochrome P450s,[24] increased methoxyflurane defluorination and nephrotoxicity in animals in previous investigations.[11–13] Specifically tested was the hypothesis that

phenobarbital would induce methoxyflurane toxicity and metabolism to MDFA and fluoride. Methoxyflurane alone caused weight loss (7% at 24 h *vs.* baseline, not shown), decreased urine osmolality, and minimal diuresis or change in BUN (fig. 2). There were also only minor changes in renal histology, with occasional mild swelling and vacuolization of focal clusters of proximal tubules noted in some animals. Phenobarbital induction enhanced methoxyflurane effects on renal function, including increased diuresis, BUN, and decreased urine osmolality (fig. 2). Baseline urine volumes in phenobarbital-treated rats were higher than in controls, as observed previously.[11,12,20] Phenobarbital pretreatment further increased total 4-day urine output after methoxyflurane, from 38 ± 7 to 53 ± 5 ml ($P < 0.05$; fig. 2). Two of five phenobarbital-pretreated rats had modestly greater histologic injury than those treated with methoxyflurane alone (which showed little effect). This injury included widespread tubular swelling and vacuolation, tubular dilation, and rare, focal, tubular necrosis. There was, however, no statistical difference between groups (median score 0.2 *vs.* 0.5 in controls and phenobarbital-treated rats).

After methoxyflurane anesthesia, inorganic fluoride and MDFA were both readily excreted in urine, although no DCAA was detectable (fig. 3). Phenobarbital induction decreased urine MDFA excretion and had minimal effect on daily urine fluoride excretion. Total 0- to 96-h urine MDFA excretion was 130 ± 17 *versus* 204 ± 45 μmol in controls ($P < 0.05$), and total 0- to 96-h urine fluoride excretion was 122 ± 7 *versus* 112 ± 20 μmol in controls (not significant). No DCAA was detectable in the urine of control or phenobarbital-induced rats anesthetized with methoxyflurane.

Experiments were conducted to determine the effect of MDFA on renal function, and the influence of phenobarbital on MDFA metabolism and renal function. Specifically tested was the hypothesis that MDFA is nephrotoxic, or that phenobarbital would increase MDFA toxicity (and possibly metabolism). Administration of 0.225 mmol/kg MDFA followed by 0.112 mmol/kg on the next 3 days had no effect on animal weight (not shown), nor on BUN, urine volume, or urine osmolality (fig. 4). Higher doses of MDFA (as high as 1.9 mmol/kg, then 0.9 mmol/kg on the next 3 days) also had no significant effect on urine volume, urine osmolality, or BUN concentrations, and there was no histologic evidence of renal toxicity.[25] Phenobarbital pretreatment did not alter the response to MDFA (fig. 4), and no renal histologic abnormalities were observed in rats treated with phenobarbital and MDFA. Total 4-day urine output after MDFA was 23 ± 7 ml in the phenobarbital-treated group *versus* 23 ± 7 in those receiving MDFA alone (not significant).

The pattern of metabolite excretion after MDFA administration is shown in figure 5. MDFA was excreted in urine, in small amounts. Fluoride was also detected in urine, albeit also in low amounts. DCAA was not detected in urine. After administration of approximately

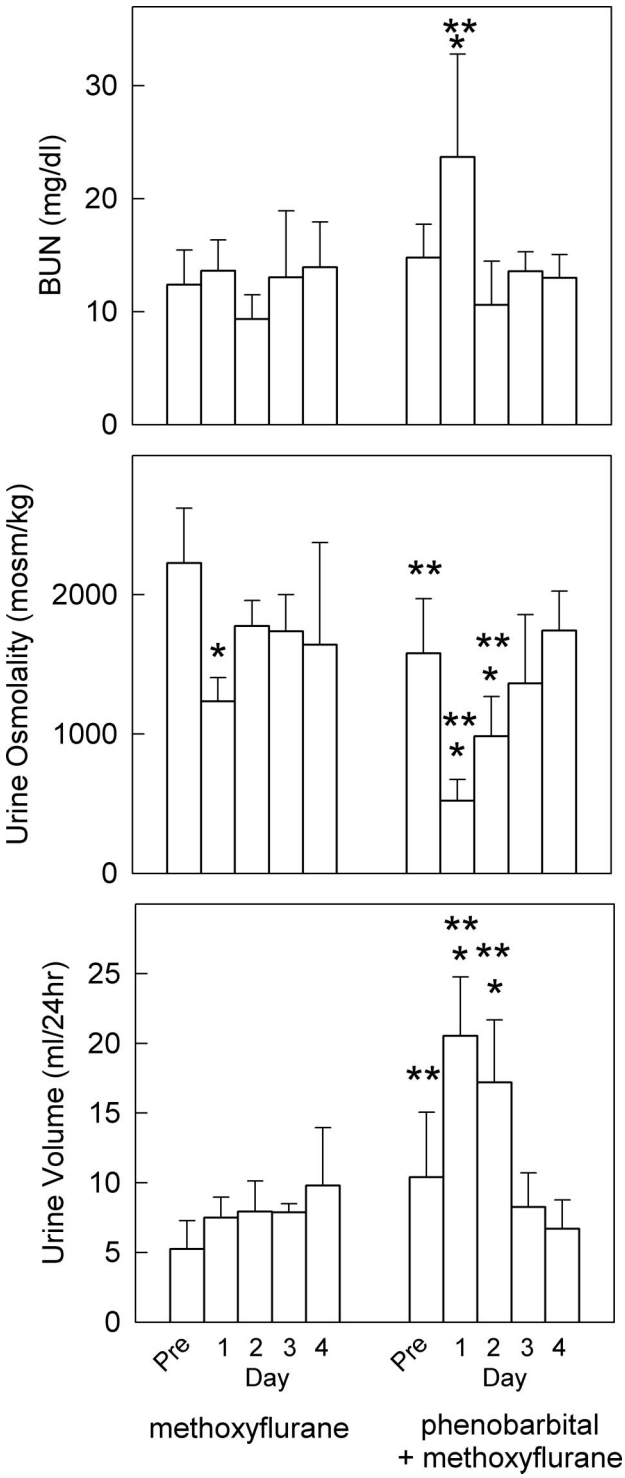

Fig. 2. Influence of phenobarbital pretreatment on methoxyflurane renal effects. Results are mean ± SD (controls n = 4, phenobarbital treated n = 5). Each *set of five bars* represents the preanesthetic sample and days 1–4 after methoxyflurane anesthesia. * Significantly different from premethoxyflurane sample ($P < 0.05$). ** Significantly different from methoxyflurane controls ($P < 0.05$). BUN = blood urea nitrogen.

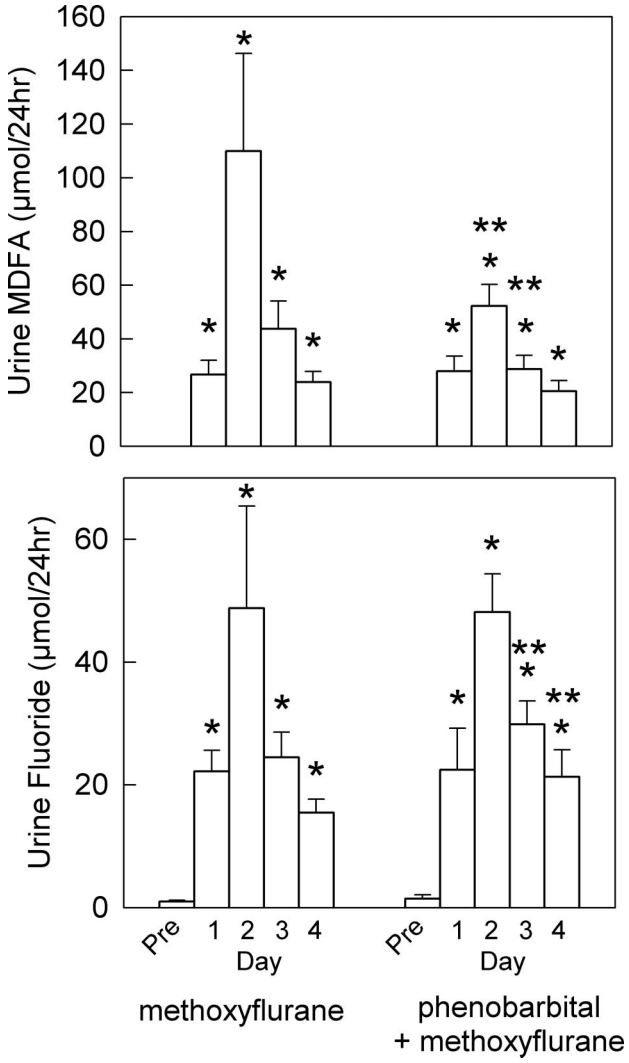

Fig. 3. Influence of phenobarbital pretreatment on methoxyflurane metabolism *in vivo*. Results are urine methoxyflurane metabolite excretion (mean ± SD; controls n = 4, phenobarbital treated n = 5). Each *set of five bars* represents the preanesthetic sample and days 1–4 after methoxyflurane anesthesia. No dichloroacetic acid was detected in any urine sample (results not shown). No methoxydifluoroacetic acid (MDFA) was detected in the preanesthetic urine. * Significantly different from premethoxyflurane sample ($P < 0.05$). ** Significantly different from methoxyflurane controls ($P < 0.05$).

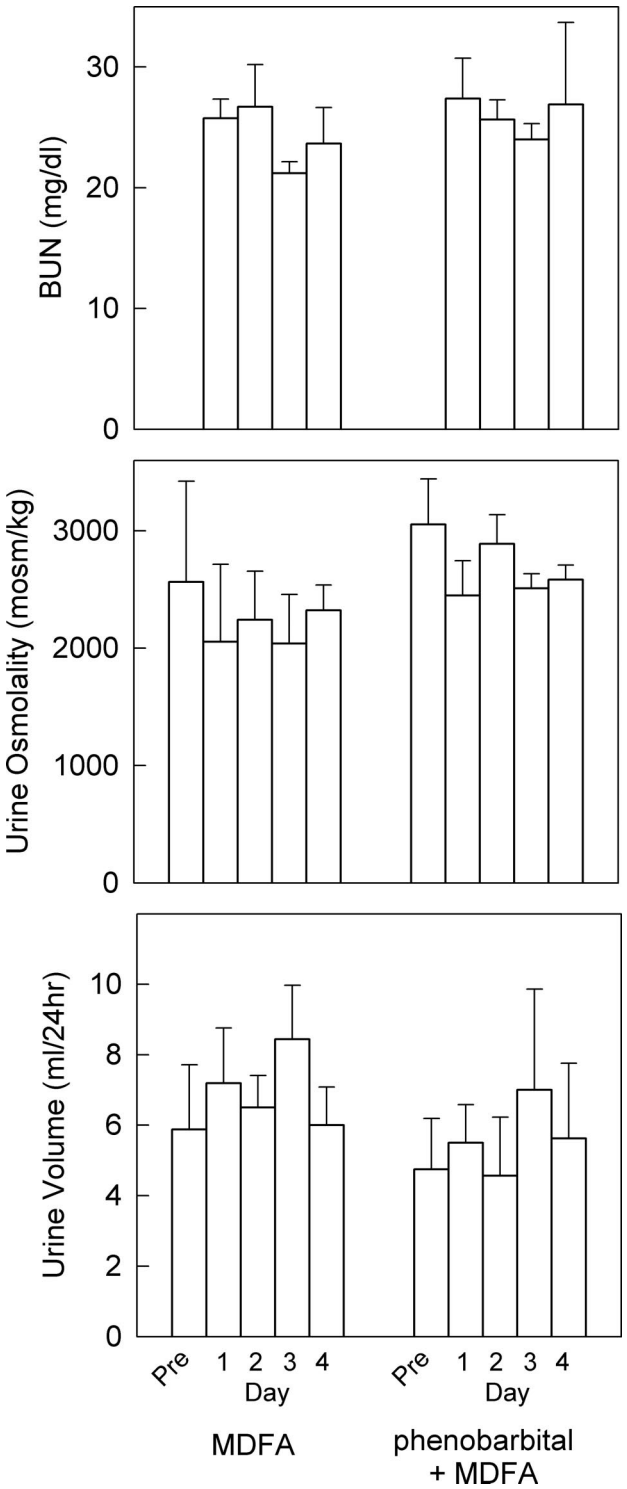

Fig. 4. Influence of phenobarbital pretreatment on methoxydifluoroacetic acid (MDFA) renal effects. Results are mean ± SD (n = 4/group). Each *set of five bars* represents the preanesthetic sample and days 1–4 after MDFA. There were no significant differences between the pre- and post-MDFA samples in either group or between phenobarbital-treated and saline-treated controls. BUN = blood urea nitrogen.

140 μmol MDFA over 4 days, cumulative metabolite recovery in controls was 49 ± 6 μmol MDFA and 22 ± 2 μmol fluoride. Phenobarbital had negligible effect on MDFA metabolism or excretion (fig. 5). Cumulative metabolite recovery after phenobarbital pretreatment was 44 ± 6 μmol MDFA and 17 ± 3 μmol fluoride, which was not different from that in rats receiving MDFA alone.

To test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity, the effect of barium sulfate on methoxyflurane metabolism and nephrotoxicity was evaluated. Pretreatment with barium sulfate diminished the diuresis, decrease in urine osmolality, and increase in BUN caused by methoxyflurane (fig. 6). Total 4-day urine output was 20 ± 9 ml in the pretreated group *versus* 35 ± 9 ml in rats receiving methoxyflurane alone ($P < 0.05$). The pattern of normal to mild histologic change seen with methoxyflurane was not altered

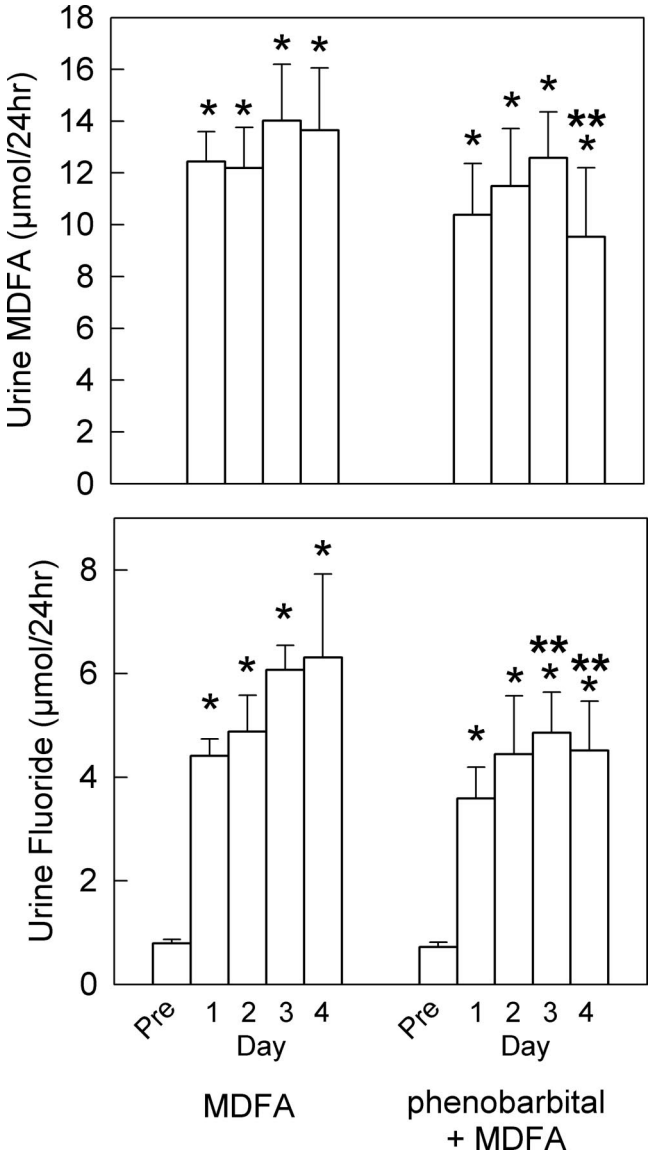

Fig. 5. Influence of phenobarbital pretreatment on methoxydifluoroacetic acid (MDFA) metabolism *in vivo*. Results are urine MFDA metabolite excretion (mean ± SD, n = 4/group). Each *set of five bars* represents the preanesthetic sample and days 1–4 after MDFA. No dichloroacetic acid was detected in any urine sample (results not shown). * Significantly different from pre-MDFA sample ($P < 0.05$). ** Significantly different from MDFA controls ($P < 0.05$).

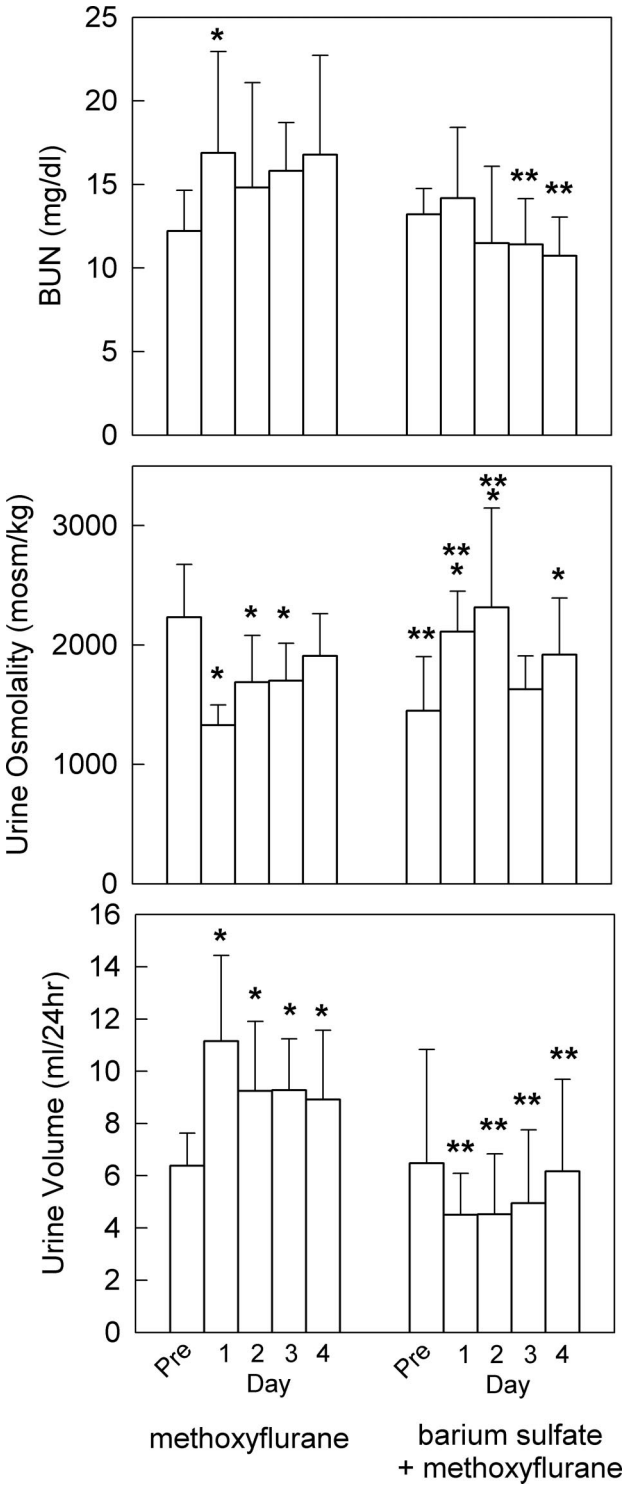

Fig. 6. Influence of barium sulfate pretreatment on methoxyflurane renal effects. Results are mean ± SD (controls n = 8, barium sulfate treated n = 10). Each *set of five bars* represents the preanesthetic sample and days 1–4 after methoxyflurane anesthesia. * Significantly different from premethoxyflurane sample ($P < 0.05$). ** Significantly different from methoxyflurane controls ($P < 0.05$). BUN = blood urea nitrogen.

by pretreatment with barium sulfate. Barium sulfate pretreatment decreased methoxyflurane metabolism, evidenced by diminished urine MDFA and fluoride excretion (fig. 7). Total 0- to 96-h urine MDFA excretion was 114 ± 24 *versus* 252 ± 40 μmol in controls ($P < 0.05$), and total 0- to 96-h urine fluoride excretion was 60 ± 15 *versus* 98 ± 27 μmol in controls ($P < 0.05$). No DCAA was detectable in the urine of either control or barium sulfate–pretreated rats anesthetized with methoxyflurane.

Experiments were conducted to determine the effect of phenobarbital and barium sulfate pretreatment on the hepatic and renal metabolism of methoxyflurane and MDFA *in vitro* (table 1). Phenobarbital induction significantly increased hepatic microsomal methoxyflurane metabolism. In contrast, there was no evidence for

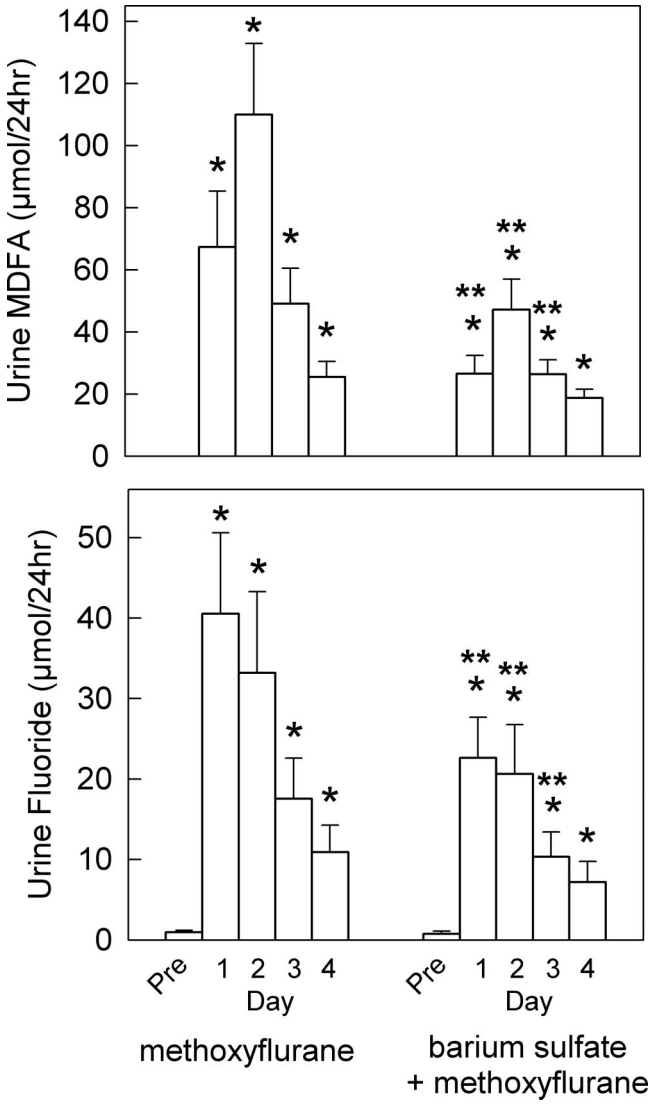

Fig. 7. Influence of barium sulfate pretreatment on methoxyflurane metabolism *in vivo*. Results are the urine metabolite excretion (mean ± SD; controls n = 8, barium sulfate treated n = 10). Each *set of five bars* represents the preanesthetic sample and days 1–4 after methoxyflurane anesthesia. No dichloroacetic acid was detected in any urine sample (results not shown). * Significantly different from premethoxyflurane sample ($P < 0.05$). ** Significantly different from methoxyflurane controls ($P < 0.05$). MDFA = methoxydifluoroacetic acid.

MDFA metabolism by liver microsomes from control rats and no effect of phenobarbital induction (*i.e.*, no metabolism), based on the formation of MDFA and fluoride. No DCAA formation from MDFA was observed. Barium sulfate pretreatment had no effect on renal microsomal methoxyflurane metabolism, and decreases in hepatic microsomal methoxyflurane metabolism were not statistically significant ($P = 0.08$ for MDFA).

Discussion

Methoxyflurane metabolism clearly results in nephrotoxicity. The two major routes of methoxyflurane metabolism, O-demethylation and dechlorination, yield inorganic fluoride and DCAA (further metabolized to glyoxylate and oxalic acid), and MDFA, respectively. MDFA can also yield inorganic fluoride, thought to result from either decomposition or further metabolism.[8] Thus, both metabolic pathways may theoretically generate fluoride, whose formation is positively associated with nephrotoxicity. Nevertheless, the metabolic pathway responsible for methoxyflurane nephrotoxicity has never been identified. This investigation attempted to identify which pathway of methoxyflurane metabolism mediates nephrotoxicity in rats.

Phenobarbital pretreatment induced hepatic methoxyflurane metabolism *in vitro* to both inorganic fluoride and MDFA and increased methoxyflurane nephrotoxicity *in vivo*. In contrast, phenobarbital had no effect on MDFA metabolism *in vitro*, no effect on MDFA metabolism *in vivo*, and no influence on the renal effects of MDFA *in vivo*. Indeed, MDFA seemed not to undergo metabolism *in vitro* or *in vivo* and had no effect on renal function under any condition. Together, these results suggest that phenobarbital effects on methoxyflurane nephrotoxicity are not attributable to induction of the dechlorination pathway, an increase in MDFA formation, toxicity of MDFA, or induction of MDFA metabolism to another toxic metabolite. Rather, these results support the hypothesis that phenobarbital effects on methoxyflurane nephrotoxicity are due to induction of the O-demethylation pathway.

What is the source and significance of increased inorganic fluoride after methoxyflurane? The relative lack of MDFA metabolism and defluorination *in vitro* and *in vivo* is consistent with previous observations showing only small amounts of fluoride formation after MDFA administration, which was attributed to nonenzymatic degradation of MDFA after excretion in urine.[19] Thus, inorganic fluoride formation from methoxyflurane seems to arise primarily from the O-demethylation pathway, rather than the dechlorination pathway.

These results imply that well-described associations *in vivo* between increased (or decreased) methoxyflurane metabolism, increased (or decreased) plasma and urine fluoride concentrations, and increased (or decreased) methoxyflurane nephrotoxicity, in animals and humans, are attributable to an underlying association between renal toxicity and methoxyflurane O-demethylation. Although dechlorination was quantitatively greater than O-demethylation, in agreement with previous results,[19] O-demethylation seems to be the toxic pathway.[26]

Several metabolites arise from methoxyflurane O-demethylation, including the putative unstable compounds dichlorodifluoroethanol and dichloroacetyl fluoride (which may acylate tissue macromolecules) and the stable metabolites inorganic fluoride, DCAA, and its subsequent metabolites glyoxylate and oxalic acid. One or more of these metabolites may be implicated in me-

Table 1. Effect of Phenobarbital and Barium Sulfate on the Hepatic Microsomal Metabolism of Methoxyflurane and MDFA

			Metabolite Formation, nmol/mg protein		
Substrate	Animal Treatment	Tissue	F	MDFA	DCAA
Methoxyflurane	Control	Liver (n = 4)	4.1 ± 0.7	3.2 ± 0.4	1.5 ± 0.1
	Phenobarbital	Liver (n = 4)	10.4 ± 0.7*	15.8 ± 0.9*	2.5 ± 0.2*
MDFA	Control	Liver (n = 4)	1.5 ± 2.2		0.0 ± 0.0
	Phenobarbital	Liver (n = 4)	16.0 ± 2.9	†	0.0 ± 0.0
Methoxyflurane	Control	Liver (n = 4)	4.9 ± 0.7	5.5 ± 1.3	1.6 ± 0.2
	Barium sulfate	Liver (n = 3)	3.4 ± 0.9	3.8 ± 0.3	1.2 ± 0.6
Methoxyflurane	Control	Kidney (n = 3)	0.8 ± 0.5	ND	
	Barium sulfate	Kidney (n = 3)	0.7 ± 0.1		

* Significantly different from untreated control ($P < 0.05$). † Residual methoxydifluoroacetic acid (MDFA) concentrations were not different between phenobarbital-treated and control animals.

DCAA = dichloroacetic acid; ND = not determined.

thoxyflurane nephrotoxicity. Inorganic fluoride can cause nephrotoxicity, albeit at high doses.[9,10,12] For example, renal changes similar to those after methoxyflurane were replicated by injecting 100 μmol sodium fluoride, but the accompanying serum fluoride concentrations (400 μM) were relatively high.[10] DCAA and other haloacids are renal mitochondrial toxins and can cause nephrotoxicity.[27,28] Oxalic acid was previously discounted as the causative metabolite in methoxyflurane nephrotoxicity.[8,12] It is clear, however, that coformation of inorganic fluoride along with, or from, these other metabolites implies that measured fluoride concentrations may reflect the formation of one or more of these other, potentially toxic, metabolites. The(ir) identity, however, remains unknown. The effects of other methoxyflurane metabolites, alone or in combination, is unknown. Identification of the nephrotoxic methoxyflurane metabolite(s) requires further investigation.

In addition to metabolite-specific considerations, there may be tissue-specific considerations. A previous investigation showed that human kidney microsomes substantially defluorinated methoxyflurane but not the nonnephrotoxic anesthetic sevoflurane.[15] This suggested that an intrarenally generated toxic metabolite (fluoride or others) may contribute to methoxyflurane nephrotoxicity, and the relative paucity of renal defluorination of other volatile anesthetics may explain the absence of their nephrotoxicity, despite plasma fluoride concentrations which may exceed 50 μM.[15]

The ideal experiment to test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity would selectively manipulate renal P450 activity and metabolism, without altering hepatic metabolism or systemic fluoride concentrations. Based on reports of selective induction of renal P450 and suppression of hepatic P450 by fibrates and particulate irritants,[22,29,30] such an experiment was attempted. Preliminary studies with hepatic and renal microsomes from rats treated with ciprofibrate, clofibrate, celite, or barium sulfate characterized their effects on methoxyflurane metabolism. Unfortunately, no regimen resulted in both renal induction and hepatic inhibition of methoxyflurane metabolism (not shown), although barium sulfate did result in mild down-regulation of hepatic microsomal methoxyflurane metabolism without a significant alteration in renal microsomal methoxyflurane metabolism. Therefore, barium sulfate effects on methoxyflurane metabolism and toxicity were evaluated *in vivo*. Both dechlorination and O-demethylation were inhibited, and methoxyflurane toxicity was prevented. These results suggest that hepatic methoxyflurane metabolism does impact methoxyflurane nephrotoxicity, although they do not inform on the hypothesis regarding renal metabolism. Nevertheless, it is interesting to note that in a previous investigation, phenobarbital induction (and enzyme inhibition) markedly increased (and decreased) the concentration of fluoride, and presumably other methoxyflurane metabolites in kidneys, said to be consistent with enhanced (and inhibited) renal methoxyflurane O-demethylation.[31]

Results of the current investigation can be compared with those reported previously. The identity of MDFA as the organic acid methoxyflurane metabolite in urine, established here using mass spectrometry, confirms the identification by nuclear magnetic resonance spectroscopy.[18,19] Effects of methoxyflurane anesthesia, including weight loss, diuresis, and decrease in urine osmolality, were similar to those seen by Mazze *et al.*[9,10,12,20,32,33] The absence of necrosis under light microscopy with 0.5% methoxyflurane for 3 h is similar to that reported previously.[20,34] The extent of methoxyflurane metabolism and toxicity (diuresis, osmolality) was, however, less than that reported previously. This seems related to age. We used male Fischer 344 rats (3 months old, 250–300 g), whereas Mazze *et al.*[9–12,20] used much older (6- to 12-month-old) rats. Older rats (12 months *vs.* 6 weeks) had greater methoxyflurane blood concentrations, greater methoxyflurane defluorination as evidenced by greater serum inorganic fluoride concentrations (37 ± 5 *vs.* 126 ± 23 μM) and urine fluoride excretion (0.1 *vs.* 0.3

μmol/g body weight) in rats, and greater diuresis in response to methoxyflurane.[32] Phenobarbital induction of hepatic microsomal methoxyflurane defluorination was similar to the increase previously reported.[11] Phenobarbital effects on methoxyflurane toxicity were also consistent with previous reports.[11,12,33] In the current investigation, urine fluoride excretion was unchanged in phenobarbital-induced rats, but urine MDFA excretion was decreased. Mazze *et al.*[11] reported that urine fluoride excretion was increased in phenobarbital-induced rats, but urine organic fluoride (presumably MDFA) excretion was unchanged. These results are not qualitatively different, given the age differences in the rats and the implications for metabolism, and because the rats in the current investigation received 0.5% methoxyflurane for 3 h whereas those in the previous investigation received 0.25% methoxyflurane for 1 h, and methoxyflurane metabolism is dose dependent and saturable at higher doses.[35] Apparent lack of MDFA metabolism *in vitro* or *in vivo* is consistent with previous reports.[18,26]

Because methoxyflurane disappeared from clinical use decades ago, why are these findings important? They may have mechanistic and clinical implications. Methoxyflurane causes nephrotoxicity as a result of metabolism, and methoxyflurane toxicity is associated with plasma fluoride concentrations of greater than 50 μM, but other volatile anesthetics have little or no effect on renal function under conditions in which plasma fluoride concentrations exceed 50–100 μM. This remained previously unexplained. Methoxyflurane O-demethylation, the apparent pathway of toxification, generates inorganic fluoride as well as many other metabolites. If one of these other metabolites that are unique to methoxyflurane, alone or in concert with fluoride, mediates methoxyflurane toxicity, this may provide an apparent explanation for the selective nephrotoxicity of methoxyflurane but not other volatile anesthetics. If so, exclusive focus on plasma fluoride concentrations as a harbinger of renal toxicity would seem unfounded. Furthermore, with regard to anesthetic development, the use of *in vitro* assays that evaluate defluorination as the primary screen for potential *in vivo* toxicity merits reevaluation.

In summary, these results suggest that neither MDFA nor methoxyflurane metabolism to MDFA is nephrotoxic, and that phenobarbital exacerbation of methoxyflurane nephrotoxicity in rats is not attributable to enhanced methoxyflurane dechlorination or increased MDFA formation. Results suggest that inorganic fluoride arises primarily from methoxyflurane O-demethylation, that phenobarbital exacerbation of methoxyflurane nephrotoxicity is attributable to enhanced methoxyflurane O-demethylation, and that inhibition of methoxyflurane O-demethylation decreases nephrotoxicity. Together, these results suggest that O-demethylation is the mechanism of methoxyflurane nephrotoxicity. Because inorganic fluoride is one of many metabolites arising from O-demethylation, it may be responsible for methoxyflurane toxicity and/or simply reflecting the formation of a different toxic metabolite. These results may have implications for the interpretation of clinical anesthetic defluorination, use of volatile anesthetics, and the laboratory methods used to evaluate potential anesthetic toxicity.

References

1. Artusio JF, Van Poznak A, Hunt RE, Tiers FM, Alexander M: A clinical evaluation of methoxyflurane. ANESTHESIOLOGY 1960; 21:512–7
2. Crandall WB, Pappas SG, Macdonald A: Nephrotoxicity associated with methoxyflurane anesthesia. ANESTHESIOLOGY 1966; 27:591–607
3. Mazze RI: Fluorinated anaesthetic nephrotoxicity: An update. Can Anaesth Soc J 1984; 31:S16–22
4. Wallin RF, Regan BM, Napoli MD, Stern IJ: Sevoflurane: A new inhalational anesthetic agent. Anesth Analg 1975; 54:758–66
5. Patel SS, Goa KL: Sevoflurane: A review of its pharmacokinetic and pharmacodynamic properties and its clinical use in general anesthesia. Drugs 1996; 51:658–700
6. Holaday DA, Rudofsky S, Treuhaft PS: The metabolic degradation of methoxyflurane in man. ANESTHESIOLOGY 1970; 33:579–93
7. Taves DR, Fry BW, Freeman RB, Gillies AJ: Toxicity following methoxyflurane anesthesia: II. Fluoride concentrations in nephrotoxicity. JAMA 1970; 214: 91–5
8. Mazze RI, Trudell JR, Cousins MJ: Methoxyflurane metabolism and renal dysfunction: Clinical correlation in man. ANESTHESIOLOGY 1971; 35:247–52
9. Mazze RI, Cousins MJ, Kosek JC: Strain differences in metabolism and susceptibility to the nephrotoxic effects of methoxyflurane in rats. J Pharmacol Exp Ther 1973; 184:481–8
10. Mazze RI, Cousins MJ, Kosek JC: Dose-related methoxyflurane nephrotoxicity in rats: A biochemical and pathologic correlation. ANESTHESIOLOGY 1972; 36:571–87
11. Mazze RI, Hitt BA, Cousins MJ: Effect of enzyme induction with phenobarbital on the *in vivo* and *in vitro* defluorination of isoflurane and methoxyflurane. J Pharmacol Exp Ther 1974; 190:523–9
12. Cousins MJ, Mazze RI, Kosek JC, Hitt BA, Love FV: The etiology of methoxyflurane nephrotoxicity. J Pharmacol Exp Ther 1974; 190:530–41
13. Baden JM, Rice SA, Mazze RI: Deuterated methoxyflurane anesthesia and renal function in Fischer rats. ANESTHESIOLOGY 1982; 56:203–6
14. Cousins MJ, Mazze RI: Methoxyflurane nephrotoxicity: A study of dose response in man. JAMA 1973; 225:1611–6
15. Kharasch ED, Hankins DC, Thummel KE: Human kidney methoxyflurane and sevoflurane metabolism: Intrarenal fluoride production as a possible mechanism of methoxyflurane nephrotoxicity. ANESTHESIOLOGY 1995; 82:689–99
16. Brown BR Jr: Shibboleths and jigsaw puzzles: The fluoride nephrotoxicity enigma. ANESTHESIOLOGY 1995; 82:607–8
17. Mazze RI, Jamison R: The renal effects of sevoflurane. ANESTHESIOLOGY 1995; 83:443–5
18. Selinsky BS, Perlman ME, London RE: *In vivo* nuclear magnetic resonance studies of hepatic methoxyflurane metabolism: II. A reevaluation of hepatic metabolic pathways. Mol Pharmacol 1988; 33:567–73
19. Selinsky BS, Perlman ME, London RE: *In vivo* nuclear magnetic resonance studies of hepatic methoxyflurane metabolism: I. Verification and quantitation of methoxydifluoroacetate. Mol Pharmacol 1988; 33:559–66
20. Barr GA, Cousins MJ, Mazze RI, Hitt BA, Kosek JC: A comparison of the renal effects and metabolism of enflurane and methoxyflurane in Fischer 344 rats. J Pharmacol Exp Ther 1974; 188:257–64
21. Kharasch ED, Thummel KE: Identification of cytochrome P450 2E1 as the predominant enzyme catalyzing human liver microsomal defluorination of sevoflurane, isoflurane and methoxyflurane. ANESTHESIOLOGY 1993; 79:795–807
22. Sewer MB, Koop DR, Morgan ET: Differential inductive and suppressive effects of endotoxin and particulate irritants on hepatic and renal cytochrome P-450 expression. J Pharmacol Exp Ther 1997; 280:1445–54
23. Sheffels P, Schroeder JL, Altuntas TG, Liggitt HD, Kharasch ED: Role of cytochrome P4503A in cysteine S-conjugates sulfoxidation and the nephrotoxicity of the sevoflurane degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether ("compound A") in rats. Chem Res Toxicol 2004; 17: 1177–89
24. Lohr JW, Willsky GR, Acara MA: Renal drug metabolism. Pharmacol Rev 1998; 50:107–41
25. Kharasch ED, Schroeder JL, Liggitt HD, Park SB, Whittington D, Sheffels P: New insights into the mechanism of methoxyflurane nephrotoxicity and impli-

cations for anesthetic development (part 2): Identification of the nephrotoxic metabolites. ANESTHESIOLOGY 2006; 105:737–45

26. Gruenke LD, Konopka K, Cadieu M, Waskell L: The stoichiometry of the cytochrome P-450-catalyzed metabolism of methoxyflurane and benzphetamine in the presence and absence of cytochrome b5. J Biol Chem 1995; 270:24707–18

27. Lash LH, Xu Y, Elfarra AA, Duescher RJ, Parker JC: Glutathione-dependent metabolism of trichloroethylene in isolated liver and kidney cells of rats and its role in mitochondrial and cellular toxicity. Drug Metab Dispos 1995; 23:846–53

28. Lantum HB, Baggs RB, Krenitsky DM, Anders MW: Nephrotoxicity of chlorofluoroacetic acid in rats. Toxicol Sci 2002; 70:261–8

29. Zangar RC, Woodcroft KJ, Novak RF: Differential effects of ciprofibrate on renal and hepatic cytochrome P450 2E1 expression. Toxicol Appl Pharmacol 1996; 141:110–6

30. Cummings BS, Parker JC, Lash LH: Cytochrome P450-dependent metabolism of trichloroethylene in rat kidney. Toxicol Sci 2001; 60:11–9

31. Berman ML, Lowe HJ, Bochantin J, Hagler K: Uptake and elimination of methoxyflurane as influenced by enzyme induction in the rat. ANESTHESIOLOGY 1973; 38:352–7

32. Bell LE, Hitt BA, Mazze RI: The influence of age on the distribution, metabolism and excretion of methoxyflurane in Fischer 344 rats: A possible relationship to nephrotoxicity. J Pharmacol Exp Ther 1975; 195:34–40

33. Brodeur J, Paquin P, Authier L, Geadah D, Yamauchi M, Cote M: Influence of phenobarbital pretreatment on methoxyflurane and sodium fluoride nephropathy in Fischer 344 rats. Toxicol Appl Pharmacol 1976; 37:349–61

34. Kosek JC, Mazze RI, Cousins MJ: The morphology and pathogenesis of nephrotoxicity following methoxyflurane (Penthrane) anesthesia: An experimental model in rats. Lab Invest 1972; 27:575–80

35. Adler L, Brown BRJ, Thompson MF: Kinetics of methoxyflurane biotransformation with reference to substrate inhibition. ANESTHESIOLOGY 1976; 44:380–5